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Award Number: DAMD17-98-1-8593

TITLE: Identification of Novel Secreted Molecules Prostate Cancer

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REPORT DATE: December 2000

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20020910 038

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE December 2000	3. REPORT TYPE AND DATES COVERED Final (1 Jun 98 - 30 Nov 00)	
4. TITLE AND SUBTITLE Identification of Novel Secreted Molecules Prostate Cancer			5. FUNDING NUMBERS DAMD17-98-1-8593	
6. AUTHOR(S) Phillip Koeffler, M.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cedars-Sinai Medical Center Los Angeles, California 90048 E-MAIL: koeffler@cshs.org			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) We have developed an approach to isolate new tumor markers of prostate cancer. In order to identify genes encoding secreted protein, we have modified the signal sequence trap technique, using the SUC 2 gene encoding a secreted protein of yeast. Only the yeast which have been transformed with cDNAs containing secretory signal sequences can grow into colonies on the plates which contain sucrose as the only source of sugar. Early difficulties identifying positive clones with our initial vector system were problematic; we obtained a new vector system to reduce the possibility of enzymatically inactive fusion invertase and enhance the sensitivity of this signal trap system which allowed us to obtain positive clones. We successfully constructed and subtracted a prostate specific cDNA library. We, however, found that this technique gave too many false-positive and false-negative results. Therefore, We turned to using another molecular biology technique RDA, and we were fortunate to find TMEFF2. It is gene that we have discovered to be highly expressed in prostate cancers and to be under androgen control. It is highly expressed in AD, but not AI prostate cancer cells. Forced expression of this protein markedly decreases growth of prostate cancer cells both in vitro and in nude mice. In addition, other investigators have suggested TMEFF2 may be a tumor suppressor gene in bladder and colon cancers. Thus, loss of expression of this gene may be associated with progression to androgen independence. TMEFF2 could be a target for therapeutic manipulation of this disease and may be used as a tumor marker for early diagnosis of prostate cancer because it is a secreted protein. will continue to pursue the selection of positive clones, make RNA dot blots from various tissues to establish tissue specificity of the cDNA and perform homology searches to determine if we have found a novel gene. Unique genes will be cloned into a GST vector and fusion proteins will be made and used to make antibody against the protein. Serum sample assays will be developed from antibodies that identify prostate specific genes. Additionally, structure/function analysis and sense and antisense expression vectors will be employed to elucidate further the function of these genes				
14. SUBJECT TERMS Prostate Cancer			15. NUMBER OF PAGES 32	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT 1 Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

TABLE OF CONTENTS

Cover	Page 1
SF 298	Page 2
Table of Contents	Page 3
Introduction	Page 4
Body	Page 4
Key Research Accomplishments	Page 9
Reportable Outcomes	Page 9
Conclusions	Page 9
References	Page 9
Reprints	Page 10

Introduction: The subject of our studies was to identify secreted proteins made by prostate cancer. The purpose of the grant was to develop a unique assay of early detection of prostate cancer. The scope of the research included a prostate cancer cDNA library hooked-up to the yeast invertase cDNA with the invertase gene having a deletion of the signal sequence. This chimeric cDNA was placed in yeast that have had the endogenous invertase gene removed. The yeast were grown in agar containing sucrose, and only those yeast that contain a chimeric cDNA which codes for a signal secreted protein having a signal sequence, should grow. The yeast clones were isolated, grown-up and the recombinant genes were purified and sequenced. The interesting genes were to be further studied including development of antibodies and use of these antibodies to look at levels of the protein in serum of individuals with prostate cancer.

Body:

Task 1: Confirm preliminary experiments: As stated last year, we were able to have the yeast vector system work correctly with positive and negative controls.

Task 2: Construct and subtract a prostate specific cDNA library. As stated, we did make a cDNA library and made modifications of the yeast vector such that it contained a hybrid human serum albumin- α factor KEX2 site between the cDNA cloning site and the invertase lacking both initiation codon and signal peptide. The Hex is an endogenous yeast protease and therefore that site should be cleaved during secretion. This should result in an invertase that has complete enzymatic activity because it will be released from the fused cDNA from the cDNA library. In addition, we modified the vector by adding a synthetic linker and coding a hydrophobic amino acid stretch pSUC2T7M13ORI.

Task 3: Selection of positive clones. We obtained positive clones. When these clones were grown-up, purified and sequenced, the examination of the sequences was disappointing. We found that approximately 50% of the clones did not have signal sequences. Those that did have signal sequences, appeared to be fairly uninteresting.

Task 4: For a gene that appears to identify a prostate-specific secreted protein, an antibody will be developed to examine serum samples from appropriate individuals. We have indeed now made several heterologous antibodies against different regions of a prostate specific protein called TMEFF2, which is a gene that we cloned. We are making the antibody against several unique regions in order to be able to develop an ELISA. To understand more about TMEFF2 please see Task 5.

Task 5: The function of the novel, secreted protein will be pursued by a variety of structure-function analyses, and the use of sense and antisense expression vectors. As mentioned in Task 3, when we analyzed the inserts of the yeast, we found that approximately 50% of the cDNA inserts did not contain signal sequences. About 20% of them coded for "junk" and about 20% of them coded for uninteresting secreted proteins that were not prostate cancer specific. Therefore, a different approach was used to identifying a prostate cancer specific secreted protein, by using a technique called RDA. The androgen dependent (AD) LNCaP cell line versus two androgen independent (AI) cell lines (PC3 and

DU145) were subjected to subtractive hybridization using the Clontech PCR-Select™ cDNA subtraction kit (Clontech Laboratory, Inc. Palo Alto, CA). Several candidate genes were identified; and we focused on one clone that was highly expressed in LNCaP and undetectable in PC3 and DU145 (Fig. 1). Moreover, this gene was not detected in cell lines from a long list of other tissues (e.g. lung, liver, breast, pancreas, heart, hematopoietic cells), suggesting that it was not widely expressed (Fig 1). Sequence analysis revealed that this cDNA was identical to a gene that has recently been cloned by two groups from brain cDNA libraries and designated either TMEFF2 (1) or tomoregulin (2). Recently, two additional groups have cloned the gene and inexplicitly given the gene two additional names: TPEF for transmembrane protein containing epidermal growth factor and follistatin domains (3), and HPP1 because the gene was isolated from hyperplastic polyposis of the colon (4). In this report I will use the name TMEFF2.

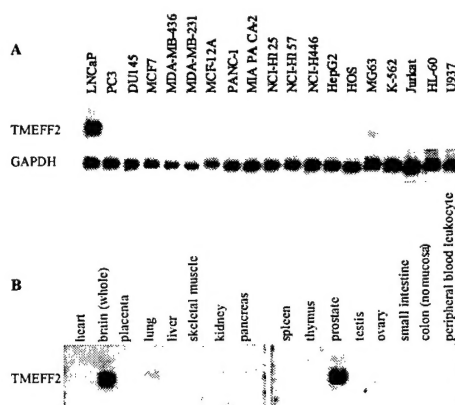


Fig. 1. Tissue specific expression of TMEFF2. A, TMEFF2 expression in cancer cell lines from various tissues. RNA was extracted from different cell lines and Northern blot was performed using radiolabeled TMEFF2 probe. The blot was rehybridized with a control $\alpha^{32}\text{P}$ -dATP-labeled GAPDH probe. B, Examination of TMEFF2 expression in multiple normal tissues. Multiple tissue Northern blots were hybridized with radiolabeled TMEFF2.

TMEFF2 is expressed predominantly in brain and prostate. It encodes a signal sequence and two follistatin-like domains and one epidermal-like domain. The presence of a signal sequence clearly suggests that the protein is either secreted or goes to the cell membrane where more than likely it can be cleaved and found in the extracellular fluid. In fact, in preliminary data, we have found it in the condition media of cells overexpressing TMEFF2 (cDNA had a histidine tag and was stably placed in LNCaP prostate cancer cell line). The EGF-like domain of TMEFF2 is highly conserved in comparison with other members of the human EGF-like family, but it has an amino acid substitution that may allow it to block signaling by the EGF receptor. The follistatin domains of TMEFF2 are also highly conserved in comparison with those of other proteins. Although the follistatin motif has been recognized in five other proteins, their function remains unclear. They may bind and inhibit growth factors such as TGF- β , activin, platelet derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (5). The TMEFF2 also has a short cytoplasmic domain with a potential G protein activating motif suggesting that TMEFF2 could act as a receptor for an extracellular ligand and mediate the signal through activation of a G protein activating motif suggesting that TMEFF2 could act as a receptor for an extracellular ligand and mediate the signal through activation of a G protein.

Preliminary Results: To test whether TMEFF2 expression is regulated by androgens, LNCaP human prostate cancer cells, which are responsive to androgens, were grown in media containing charcoal-stripped (CS) fetal bovine serum (FBS) to remove steroid hormones and treated with dihydrotestosterone (DHT). TMEFF2 mRNA levels were very low in cells grown in CS FBS and were markedly up-regulated in a dose- and time-dependent manner by DHT (Fig 2). Furthermore, we showed that an androgen-dependent xenograft known as CWR22 lost expression of TMEFF2 after castration of male animals carrying these androgen dependent prostate cancers (Fig. 2).

TMEFF2 expression in androgen-dependent(AD) and androgen-independent (AI) prostate cancer xenografts implanted in nude mice. We have begun to analyze TMEFF2 expression matched AD and AI xenografts (Fig. 3). Our results demonstrate that AD xenografts (e.g., LNCaP, CWR22), which were grown in male nude mice, expressed prominent levels of TMEFF2 mRNA. The AI xenografts (e.g. CWR22R) grown in males did not express TMEFF2. Surprisingly, the AI cells when grown in female nude mice, expressed high levels of TMEFF2 mRNA. Together with the observation that estrogen up-regulates TMEFF2 in LNCaP cells, TMEFF2 probably is regulated by estrogen in AD xenografts in female mice.

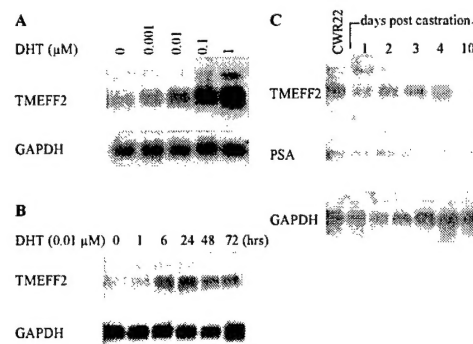


Fig. 2. TMEFF2 is regulated by androgen. A, Dose-dependent effect of dihydrotestosterone (DHT) on TMEFF2 expression. LNCaP cells were plated in media supplemented with charcoal-stripped FBS for two days, and then incubated for an additional two days with different concentrations of DHT. B, Time-course of TMEFF2 induction by DHT. LNCaP cells were plated as described in panel A and incubated with 0.01 μ M DHT for the times indicated. C, TMEFF2 expression in response to androgen withdrawal. CWR22 xenografts were propagated in male mice that were then castrated.

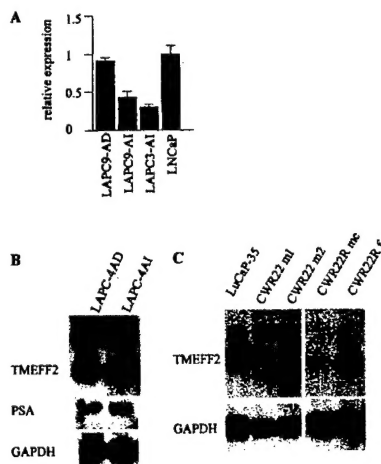


Fig. 3. Analysis of TMEFF2 expression in androgen-dependent and androgen-independent cells. A, Quantitative real time RT-PCR analysis of TMEFF2 expression in LNCaP-9 androgen-dependent/androgen-independent (AD/AI) and LNCaP-3AI xenografts. eDNAs from LNCaP-9 AD/AI and LNCaP-3AI cells were subjected to real time RT-PCR as described in Fig. 2. B, Northern blot analysis of TMEFF2 expression in LNCaP-4AD/AI xenografts. C, Northern blot analysis of TMEFF2 expression in LNCaP-35, CWR22 and CWR22R xenografts. RNA was prepared from the following samples: LNCaP-4AD propagated in a male; LNCaP-4AI propagated in a female; LNCaP-35 (androgen-dependent) propagated in a male; CWR22 (androgen-dependent) propagated in males (m1, m2); CWR22R (androgen-independent) propagated in a castrated male (mc) and CWR22R (androgen-independent) propagated in a female (f). The samples were analyzed by Northern blot hybridization with α^{32} P-dATP-labeled TMEFF2. The blots were reprobed with radiolabeled PSA (B) and GAPDH (B, C) probes.

In experiments using the CWR22 AD xenografts growing in male nude mice, castration of animals resulted in TMEFF2 levels decreasing by day 10, suggesting androgens regulate TMEFF2 expression in the mice (Fig. 2C). Also in preliminary experiments, we examined PSA levels in CWR22 (AI) and CWR22R (AD) tumors grown in male and female mice \pm castration. Unlike TMEFF2, PSA expression was restricted to the AD CWR22 cells in non-castrated males; and it was down-regulated in the CWR22R AI cells, regardless of whether they were grown in male or female mice. These results suggest that both PSA and TMEFF2 are regulated in parallel by androgen if the tumor is

AD, but they might be regulated differently by estrogen, and if the prostate cancer becomes AI, the levels of TMEFF2 may decrease while levels of PSA may increase. Using real-time PCR, we found differential expression of TMEFF2 in AD and AI matched human prostate cancer xenografts. TMEFF2 expression was 10-fold higher in LNCaP9 AD compared to LNCaP9 AI and 11-fold higher in LNCaP4 AD compared to LNCaP4 AI (Fig. 3). In the LNCaP3 AI xenograft, the TMEFF2 level was reduced in comparison to both LNCaP9 AD/AI and LNCaP4 AD/AI (Fig. 3). Furthermore, we looked at expression TMEFF2 in the xenograft LUCAP-35 which is androgen dependent and propagated in a male and it had prominent

expression of TMEFF2. Also, the CWR22 had prominent expression of TMEFF2, but a resistant androgen-independent CWR22 has low expression of TMEFF2. These cells had more prominent expression when grown in a female (Fig. 3, panel C).

Androgens and other growth factors control the proliferation of prostate cells. Members of the EGF-like family have been implicated in various autocrine loops in prostate cancer cells (6). For example, TGF- β is involved in the development of prostate cancer in animal models (7) and is highly expressed in primary and metastatic prostate cancers (8-10). Using similar conditions, we examined the effect of different hormones and growth factors on TMEFF2 expression (Fig. 4). Preliminary studies showed

that 17 β -estradiol (E2), like DHT, up-regulates levels of TMEFF2 mRNA. The androgen receptor (AR) in some prostate cancer cells (including LNCaP) can bind other steroid hormones in addition to androgens (11-14) providing a possible mechanism for the effect of estrogen on TMEFF2 transcription. Using the GeneBridge 4 Radiation hybrid Panel (Research Genetics, Inc.), we mapped the genomic locus of TMEFF2 to human chromosome 2q32. Our data suggest that TMEFF2 is prominently expressed in normal prostates and AD prostate cancers, but its expression is decreased in AI prostate cancers. TMEFF2 may behave as a tumor suppressor gene resisting the progression of AD to AI prostate cancer.

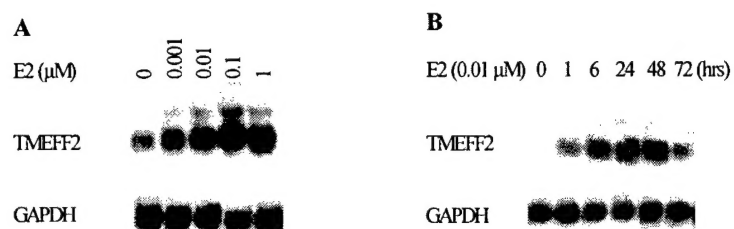


Fig. 4. Effect of 17 β -estradiol (E2) on TMEFF2 expression in LNCaP cells. *A*, Dose-dependent effect of E2 on TMEFF2 expression. *B*, Time course of TMEFF2 induction by E2. LNCaP cells were treated with either VD_3 as described in Fig 4. Northern blots were hybridized with $\alpha^{32}P$ -dATP-labeled TMEFF2, and reprobbed with radiolabeled GAPDH.

Possible use of TMEFF2 as a biomarker. TMEFF2 is a secreted protein. The extracellular domain of TMEFF2 encoding amino acid 17-150 and 151-336 have been isolated using PCR and cloned into the bacterial expression vector GEX-5X-1. The GST-TMEFF2 fusion proteins were expressed in bacteria, purified on a glutathione column and used to generate two antibodies against TMEFF2. The antibodies were made by injecting the two fragments mixed with Freund's adjuvant into rabbits. The immune sera will be concentrated, run over a protein A column and then on a TMEFF2-affinity column. Both antibodies will be tested on Western blot. We will develop an ELISA assay using both antibodies and examine sera from normal individuals, as well as those with benign prostatic hypertrophy or prostate cancer in order to determine if the ELISA can be used for detection of prostate cancer. We acknowledge that TMEFF2 is made by normal prostate cells; but similar to PSA, high expression of this protein will be associated with androgen-dependent prostate cancer. The use of two measurements, levels of serum PSA and serum TMEFF2, would probably help decrease the number of false-positive and false-negative results when attempting to differentiate prostate cancer from non-malignant conditions. Furthermore, in the setting of post-radical prostatectomy with curative intent, the TMEFF2 level should go to 0. A rise in serum TMEFF2 levels in conjunction with a rise in PSA could be used to monitor for the recurrence of prostate cancer. In addition, progression to androgen-independent prostate cancer might be identified earlier by noting a fall in the level of TMEFF2 associated with a stable or rising level of PSA in the serum.

Test the effect of overexpression of TMEFF2 on proliferation of prostate cancer cells.

Data shown in Fig. 5 provides some evidence that TMEFF2 can act as a tumor suppressor gene. Panel A shows experiments where we stably placed TMEFF2 in DU145 cells (TM1, TM2, TM3, TM4) and showed that those DU145 cells that stably expressed TMEFF2 had less proliferation than the DU145 cells not expressing TMEFF2. Panel B shows that the clonogenic growth of PC3 that are stably expressing TMEFF2 in soft-agar is less than wild-type PC-3 cells (Fig. 5). Our initial results suggest that overexpressing TMEFF2 has an inhibitory effect on cell growth. Preliminary experiments in nude mice suggest that PC-3 cells with forced expression of TMEFF2 have a markedly reduced ability to form tumors compared to the PC-3 prostatic cancer cells that do not express the TMEFF2.

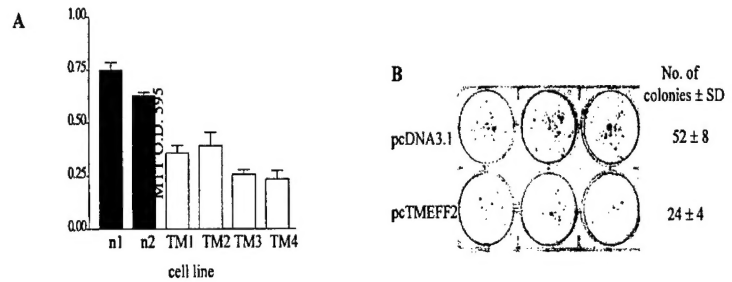


Fig. 5. Effect of TMEFF2 on growth of prostate cancer cells. *A*, MTT assay. Two DU145/neo sublines (n1, n2) and four DU145/TMEFF2 sublines (TM1-4) were plated in 96-well plates at a concentration of 1000 cells per well. MTT assays were performed after four days. Data represent mean absorbance reading at OD 590 ± S.D. Results are from quadruplicate samples (representative experiment from three independent experiments). *B*, Colony formation assay. PC3 cells were transfected with either an empty pcDNA3.1 vector or a pcDNA3.1 expressing TMEFF2 (pcTMEFF2) and cultured in the presence of G418 (500 µg/ml). The values are the mean ± SD of three plates (representative experiment from two independent experiments).

Very Recent Data:

- 1.) We have done DNA microarray analysis on PC-3 cells that do and do not overexpress TMEFF2 cells in order to identify target genes for TMEFF2. This microarray data are now being carefully analyzed to determine which targets will undergo validation and further scrutiny.
- 2.) A familial tumor suppressor gene on chromosome 8 (NKX 3.1) is poorly expressed in familial prostate cancer. In preliminary experiments using the TMEFF2 promoter hooked to a reporter gene, the NKX3.1 can upregulate expression of the TMEFF2 promoter reporter. This is extremely exciting; it suggests that TMEFF2 is a potential target of this transcription factor, and mutated expression of this transcription factor can potentially lower the expression of TMEFF-2 (also a putative tumor suppressor gene).
- 3.) In analyzing the promoter region of TMEFF2, we have identified some interesting DNA binding motifs. Taking advantage of this assessment, we have shown using reporter gene studies that c-myc can down-regulate reporter activity. This would be consistent with prostate cancer having increased expression of c-myc and decrease expression of TMEFF2 as the disease progresses. Besides doing reporter gene assays, we are stably placing a c-myc expression vector into LNCaP cells and will look at alteration of expression of TMEFF2 with induction of expression of c-myc. Furthermore, we will isolate the myc binding region of the promoter and do mutational reporter gene studies as well as perform gel shift analyses showing that myc indeed binds to the putative myc binding site. In addition, we will look at an array of prostate cancer samples for expression of myc and TMEFF2. This will include our xenographs that are androgen-dependent and androgen-independent. We would anticipate seeing an inverse correlation between expression

of myc and TMEFF2.

- 4.) We have cloned the TMEFF1 gene (a family member of TMEFF2) and we are: a.) examining its range of expression, b.) stably integrating the gene in several prostate cancer cell lines and looking for alterations of growth and functions of these subclones.

Key Research Accomplishments :

We have identified a novel secreted prostate-selective protein known as TMEFF2. It is upregulated in expression by androgens in androgen-dependent but not androgen-independent prostate cancer. TMEFF2 can slow the growth of prostate cancer cells and its expression is upregulated by the tumor suppressor gene (NKX3.1) and downregulated by an oncogene (c-myc).

Reportable outcomes:

1. Gery S, Vegesna V, Sawyers CL, Agus DB, and Koeffler HP. TMEFF2 is a New Member of the EGF-Like Family Regulated by Androgen and Having Antiproliferative Effects in Prostate Cancer Cells. American Association for Cancer Research, Vol. 42, (#3355) p. 624, March 2001.
2. Gery S, Sawyers CL, Agus DB, Said JW and Koeffler HP. TMEFF2 is an androgen-regulated gene exhibiting antiproliferative effects in prostate cancer cells. **Oncogene**, submitted, 2001.

Conclusions: We have explored the use of a yeast vector system known as pSUC2T7M13ORI; and although it gave us clones, the rate of false-positive and false-negative cDNA inserts was too high to use this technique. We have, however, cloned a gene known as TMEFF2 which has some unique properties. It is a secreted protein which is under control of androgens. To our knowledge, it is only made in two organs, the prostate and brain. TMEFF2 appears to also behave as a tumor suppressor gene; and our preliminary data suggest that it is modulated by NKX3.1, a transcription factor which has been identified as a tumor suppressor gene that is altered in a familial prostate cancer. Our preliminary data suggest that TMEFF2 expression is down-regulated by c-myc (an oncogene).

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TMEFF2 is an androgen-regulated gene exhibiting antiproliferative effects in prostate cancer cells.

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Running Title: Expression of TMEFF2 in prostate cancer cells

Key words: prostate cancer, androgen-regulated, epidermal growth factor, follistatin and antiproliferative.

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Abstract

We have identified a gene that is highly expressed in the androgen-dependent prostate cancer cell line, LNCaP. Sequence analysis revealed that it was identical to a recently cloned gene designated TMEFF2, which encodes a transmembrane protein containing an epidermal growth factor (EGF)-like motif and two follistatin domains. This gene was highly expressed only in primary samples of normal prostate and prostate cancer as well as normal brain. Expression of the gene was controlled by androgen as shown by dihydrotestosterone markedly increasing TMEFF2 expression in LNCaP cells. Also, androgen-dependent human prostate cancer xenografts (CWR22) expressed high levels of TMEFF2 and these levels markedly decreased by day 10 after castration of the mice. Furthermore, a large number of androgen-dependent xenografts (CWR22, LuCaP-35, LAPC-4AD, LAPC-9AD) exhibited higher levels of TMEFF2 mRNA than androgen-independent xenografts (CWR22R, LAPC-3AI, LAPC-4AI, LAPC-9AI). Ectopic expression of TMEFF2 in DU145 and PC3 cells resulted in their prominent inhibition of growth. Taken together, the results demonstrate that TMEFF2 is a androgen-regulated gene, which can suppress growth of prostate cancer cells and our xenograft data show that escape of prostate cancer cells from androgen modulation causes them to decrease their expression of this gene, which may result in their more malignant behavior.

Introduction

Prostate cancer initially regresses in response to antiandrogenic hormonal therapy or androgen deprivation. However, virtually all patients relapse with the emergence of androgen-independent tumor cells. The molecular basis accounting for androgen-independent cancer progression is not completely understood. In most androgen-independent prostate cancers, the androgen receptor (AR) and the androgen regulated gene prostate-specific antigen (PSA) are expressed, indicating that the AR and the downstream pathways are active in these cells despite elimination of androgen. One mechanism that has been proposed for AR activity in androgen-independent cells, is mutation in the ligand binding domain (Tilley *et al.*, 1996) or amplification of the AR gene (Visakorpi *et al.*, 1995) which would result in the ability of these cells to sustain growth at castrated levels of androgen. An alternative mechanism is ligand-independent activation of the AR. Members of non-steroidal signal transduction pathways such as insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF; Culig *et al.*, 1994), HER2/neu (Craft *et al.*, 1999) and mitogen activated protein kinase kinase (MEKK1; Abreu-Martin *et al.*, 1999) have been shown to activate the AR in the absence of androgen. However, additional genetic aberrations conferring a growth advantage independent of the AR pathway may also contribute to the growth of androgen-independent prostate cancers. Hormonal-independent cancer progression is associated with elevated autocrine production of multiple growth factors that may replace androgen as the primary regulator of growth (reviewed in Russell *et al.*, 1998).

This report identifies a new androgen regulated gene expressed almost exclusively in the prostate that is identical to the recently described TMEFF2 gene (also known as

tomoregulin), which was shown to be expressed in brain (Uchida *et al.*, 1999; Horie *et al.*, 2000). TMEFF2 encodes a transmembrane protein containing an altered EGF-like motif and two follistatin domains that can possibly serve as growth factor binding motifs (Patthy and Nikolics, 1993; Kupprion *et al.*, 1998; Patel, 1998). We show that TMEFF2 is expressed prominently in normal human prostate as well as and androgen-dependent prostate cancer cells but is down regulated in androgen-independent human prostate cancers. Furthermore, forced expression of TMEFF2 in DU145 and PC3 androgen-independent prostate cancer cell lines inhibit their growth in liquid culture. Taken together, our results suggest that TMEFF2 may play a role as an androgen-regulated protein that can suppress growth of human prostate cells and whose downregulation is associated with progression to androgen-independent growth.

Results

TMEFF2 is Highly Expressed Almost Exclusively in the Prostate. We identified the TMEFF2 gene by subtractive hybridization, and Northern blot analysis showed that it was highly expressed in the androgen-dependent prostate cancer cell line LNCaP (2 kb major band; 3.2 kb minor band) and was undetectable in the androgen-independent prostate cancer cell lines PC3 and DU145 (Fig. 1A). Moreover, this gene was not detected in cell lines from several other tissues (e.g. lung, liver, breast, pancreas, bone and hematopoietic cells, Fig. 1A). Sequence analysis revealed that this cDNA is identical to the TMEFF2 gene (data not shown). Northern blot studies of human tissues found that TMEFF2 is highly expressed only in normal prostate and brain (Fig. 1B). Using PCR based radiation hybrids the genomic locus of TMEFF2 was mapped to the human chromosome 2q32.1.

Quantitative real time RT-PCR analysis of 5 pairs of human normal and cancerous prostate primary samples showed that TMEFF2 is highly expressed in both normal and primary prostate cancer samples, similar to its expression in LNCaP cells (Fig. 2). Normal and primary cancer samples of human breast and lung were either negative or showed very low levels of TMEFF2 expression (data not shown). These results demonstrate that TMEFF2 is prominently expressed in normal prostate and primary prostate cancer but it is not highly expressed in other tissues.

TMEFF2 Expression is Regulated by Androgens. Because TMEFF2 was expressed in the androgen-dependent LNCaP cells but not in the androgen-independent PC3 and DU145 cells, we tested whether TMEFF2 is regulated by androgens. LNCaP cells were grown in media supplemented with charcoal-stripped (CS) FBS (to remove

steroid hormones) and treated with dihydrotestosterone (DHT). Levels of TMEFF2 mRNA were low in cells grown in CS FBS, and DHT increased TMEFF2 mRNA expression in a dose-dependent manner (Fig. 3A). TMEFF2 mRNA levels increased within 1 h of DHT exposure (0.01 μ M) and the highest level of expression occurred at 24 h of culture with DHT (Fig 3B).

The effect of androgen withdrawal on TMEFF2 was measured in the androgen-dependent CWR22 human prostate cancer xenografts propagated in male mice. TMEFF2 levels markedly decreased by day 10 after castration (Fig. 3C). PSA expression also decreased by day 4 after castration (Fig. 3C).

TMEFF2 Levels are Higher in Androgen-Dependent Human Prostate Cancer Xenografts than in Androgen-Independent Prostate Cancer Xenografts. Quantitative real time RT-PCR showed differential expression of TMEFF2 in LAPC-9 androgen-dependent/androgen-independent (AD/AI) human prostate cancer xenografts. TMEFF2 expression was 2.1-fold higher in LAPC-9AD compared to LAPC-9AI cells (the fold-difference was calculated as described in the Materials and Methods, Fig. 4A). TMEFF2 levels in LAPC-3AI human prostate cancer xenografts were even lower than those in the LAPC-9AI xenografts. Furthermore, Northern blot analysis showed that TMEFF2 was expressed at a higher level in the LAPC-4AD human prostate cancer xenografts compared to the LAPC-4AI xenografts (Fig. 4B). PSA levels were similar in both the LAPC-4AD and LAPC-4AI xenografts. Also, TMEFF2 was expressed at a high level in three additional androgen-dependent human prostate cancer xenografts: two CWR22 samples (m1, m2) and LuCaP-35 (Fig. 4C). TMEFF2 expression was markedly downregulated in

androgen-independent CWR22R propagated in castrated male mice. Interestingly, androgen-independent CWR22R propagated in female mice had increased levels of TMEFF2 compared to the same xenografts propagated in castrated male mice, which prompted the following studies.

17 β -estradiol (E2) and 1,25-dihydroxyvitamin D₃ (VD₃) Enhanced TMEFF2 Expression in LNCaP Cells. Genes regulated by androgen can often be induced by ligands of other nuclear hormone receptors (Veldscholte *et al.*, 1990; Hsieh *et al.*, 1996). TMEFF2 levels were upregulated in LNCaP cells by E2 in a dose- and time-dependent manner (Fig. 5A and B). The time course of TMEFF2 induction by E2 (0.01 μ M) was similar to TMEFF2 induction by DHT. Also, VD₃ induced TMEFF2 in a dose- and time-dependent manner (Figs. 5C and D). TMEFF2 mRNA levels increased after 24 h of VD₃ exposure (0.1 μ M) and continued to increase at 72 h of culture. IL-6 and EGF that were previously shown to activate the AR in an androgen-independent manner (Culig *et al.*, 1994; Chen *et al.*, 2000) did not affect TMEFF2 expression (data not shown).

Overexpression of TMEFF2 Inhibits Proliferation of Prostate Cancer Cells. The structural motifs of the TMEFF2 protein suggests that it may interact with growth factors or their receptors and can thereby effect cell growth. To test this hypothesis, we generated DU145 stable cell lines (DU145/TMEFF2) that overexpressed TMEFF2 and measured their proliferative rates. We found that the growth rate of four independent DU145/TMEFF2 clones in culture was reduced by 43-66 % as compared with DU145/neo control cell lines (transfected with the empty vector, Fig. 6A). The effect of TMEFF2 on cell proliferation was also examined by clonogenic assays. PC3 cells were

transfected with either an empty pcDNA3.1 vector or a pcDNA3.1 vector expressing TMEFF2 and these cells were cultured in the presence of G418. Expression of TMEFF2 resulted in a 34-65 % decrease in the number of G418-resistant colonies (Fig. 6B).

Discussion

This study identified a new androgen regulated gene that is identical to the TMEFF2 gene (Uchida *et al.*, 1999; Horie *et al.*, 2000). We found that TMEFF2 is highly and selectively expressed in normal brain and prostate as well as primary prostate cancer. Among a variety of cancer cell lines, TMEFF2 expression was only detected in the androgen- dependent cell line LNCaP. Consistent with these results, Liang *et al.*, also recently cloned TMEFF2 (TPEF) and showed that it was expressed predominantly in brain and prostate. By using a PCR based radiation hybrids technique, we mapped TMEFF2 to the human chromosome 2q32.1. Previously TMEFF2 was mapped by *in situ* hybridization to chromosome 2q33 (Liang *et al.*, 2000) or to chromosome 2q32.3 (Young *et al.*, 2001). The differences in TMEFF2 localization are probably due to the different methods used.

Levels of TMEFF2 in the human primary prostate cancer samples were equivalent to those found in the normal prostate. These primary cancer samples are almost assuredly androgen-dependent; in contrast, advanced metastatic prostate cancers are usually hormone-refractory. Human prostate cancer xenografts propagated in nude mice recapitulate the clinical transition from androgen-dependent to androgen-independent and facilitate the study of the pathogenesis of androgen-independent growth. We examined TMEFF2 expression following androgen withdrawal in CWR22 xenografts. Castration resulted in TMEFF2 levels that markedly decreased by day 10, suggesting that androgens regulate TMEFF2 expression. Similarly, androgen-dependent xenografts (CWR22, LuCaP-35, LAPC-4AD, LAPC-9AD) exhibited a higher level of TMEFF2 expression than androgen-independent human prostate cancer xenografts (CWR22R,

LAPC-3AI, LAPC-4AI, LAPC-9AI). Previously, several androgen-regulated genes including PSA were shown to be either similarly expressed or upregulated in recurrent androgen-independent CWR22 xenografts (Gregory *et al.*, 1998; Xu *et al.*, 2000). We have shown that PSA levels are similar in the LAPC-4AD/AI xenografts. TMEFF2 expression on the other hand, was lower in the androgen-independent cells than in the androgen-dependent cells.

In LNCaP cells, TMEFF2 was induced in a dose- and time-dependent fashion by DHT. Also, E2 and VD₃, which were shown to activate AR regulated genes (Veldscholte *et al.*, 1990; Hsieh *et al.*, 1996) induced TMEFF2 expression in LNCaP cells. The AR in LNCaP cells has a point mutation enabling it to bind estrogen in addition to androgens. E2 induction of TMEFF2 may, therefore, be regulated through the AR. Alternatively, estrogen may upregulate TMEFF2 by binding to the estrogen receptor expressed in these cells (Lau *et al.*, 2000). Of interest, TMEFF2 levels in the androgen-independent CWR22R xenografts propagated in castrated male mice were lower than the same xenografts propagated in female mice, suggesting that estrogen may have a role in regulating TMEFF2 expression.

The forced expression of TMEFF2 in DU145 and PC3 cells inhibited their proliferation in liquid culture. The EGF-like domain in TMEFF2 is conserved in comparison with other members of the EGF-like family; but in TMEFF2, a conserved arginine residue is replaced by histidine. Mutation of this arginine in the human EGF, reduced the affinity of EGF for its receptor to less than 0.5 % of normal (Engler *et al.*, 1990). Therefore, TMEFF2 is unlikely to activate the EGF receptor. Also, a previous study suggested that TMEFF2 is a putative ligand for the erbB4 receptor (Uchida *et al.*,

1999); however, erbB4 is usually not expressed in prostate cancer cells (Robinson, *et al.*, 1996). The follistatin domains of TMEFF2 are also highly conserved in comparison with those of other proteins. Follistatins are able to bind and inactivate a variety of growth factors including members of the TGF- β family, platelet-derived growth factor and vascular endothelial growth factor (Patthy and Nikolics, 1993; Kupprion *et al.*, 1998; Patel, 1998)). The function of TMEFF2 is unknown; however, based on its functional domains, we hypothesize that it might inhibit growth factors and thereby inhibit cell proliferation. Indeed, two very recent studies showed that the TMEFF2 gene is frequently hypermethylated in human tumor cells, suggesting that TMEFF2 might be a tumor suppressor gene (Liang *et al.*, 2000; Young *et al.*, 2001). Downregulation of TMEFF2 in androgen-independent prostate cancer cells may offer a growth advantage to these cells and contribute to their more aggressive phenotype compared to androgen-dependent prostate cancer cells. Of note, TMEFF2 was suggested to act as a survival factor for neurons (Horie *et al.*, 2000). Further studies are required to determine TMEFF2 role in brain and prostate.

In summary, we have shown that TMEFF2 is an androgen-regulated gene that is highly expressed in normal prostate cells and in androgen-dependent prostate cancers, but its expression is decreased in androgen-independent prostate cancers. We also found that forced expression of TMEFF2 in TMEFF2 nonexpressing human prostate cancer cells inhibited their growth. These data in concert with recent methylation studies (Liang *et al.*, 2000; Young *et al.*, 2001), suggest that TMEFF2 may act as a tumor suppressor gene in the prostate and its inactivation is associated with androgen-independent disease progression.

Materials and Methods

Prostate Cancer Specimens, Xenografts and Cell Culture. Matched pairs of normal and tumor prostate specimens were obtained from UCLA, at the time of prostatectomy; the samples were micro-dissected after histological assessment. The prostate cancer xenografts LAPC-3, -4 and -9, CWR22 and LuCaP-35 have been described (Wainstein *et al.*, 1994; Klein *et al.*, 1997; Whang *et al.*, 1998; Agus *et al.*, 1999). Androgen withdrawal was accomplished as previously described (Agus *et al.*, 1999). The following cell lines were used: prostate cancer (LNCaP, PC3 and DU145), breast cancer (MCF7, MDA-MB-436 and MDA-MB-231), normal breast (MCF-12A), pancreas cancer (PANC-1 and MIA PA CA-2), lung cancer (NCI-H125, NCI-H157 and NCI-H446), osteosarcoma (HOS and MG63), acute myeloid leukemia (HL-60 and U937), hepatoma (HepG2), chronic myelogenous leukemia (K-562) and T-lymphocyte (Jurkat), which were obtained from the American Type Culture Collection and grown in the recommended medium and conditions. For dihydrotestosterone (DHT), 17 β -estradiol (E2) or 1,25-dihydroxyvitamin D₃ (VD₃) studies, 1×10^6 LNCaP cells were seeded into 9-mm plates in RPMI with 10% FBS. After 24 h, cells were washed, re-suspended with RPMI with 10% charcoal-stripped serum and incubated for an additional 48 h. DHT, E2 or VD₃ were then added to the cells for various times and concentrations.

Northern Blot Analysis and Subtractive Hybridization. Total RNA was isolated from human prostate specimens, prostate cancer xenografts and cell lines using TRIzol (Life Technologies, Inc.). Subtractive hybridization was performed on the androgen-dependent prostate cell line LNCaP and the two androgen-independent prostate cell lines

PC3 and DU145 with the PCR-Select™ cDNA Subtraction kit (Clontech Laboratories, Inc.). The resulting products were cloned into the pGEM-T Easy vector (Promega) and their tissue specificity was determined using Northern analysis. For Northern analysis, 10 µg of total RNA was fractionated on 1.2 % agarose denaturing gels and transferred to nylon membranes (Amersham). The human multiple tissue blots were obtained from Clontech (Laboratories, Inc.). Probes were labeled with the Strip-EZ DNA kit (Ambion) and hybridizations were performed in the ULTRAhyb buffer (Ambion) according to the manufacture's instructions.

Quantitative Real Time Reverse Transcription-PCR (RT-PCR). Total RNA (2 µg) from human specimens, prostate cancer xenografts or cell lines was converted into cDNA using MMLV reverse transcriptase (Life Technologies, Inc.) according to the manufacture's instructions. The following primers and probes were used: TMEFF2 primers, 5'-GGCTGGAATTGCTCTGGTTA-3' and 5'-AGCCTGTGCGCAGGTAACA CT-3'; 18S primers, 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGA TCCTCGTTA-3'; TMEFF2 probe, 5'-TATGTGCCTGTGTGTGGCTCCAATG-3'; 18S probe, 5'-AGCAGGCGCGCAAATTACCC-3'. Primers were synthesized by Life Technologies Inc. Probes were purchased from Applied Biosystems (Foster City, CA) and were labeled with the reporter dye FAM in the 5' end and the quencher dye TAMRA in the 3' end. Amplification reactions were performed with the Universal Taqman PCR mastermix (Applied Biosystems) in triplicates in an iCycler iQ™ system (Biorad, Hercules, CA). The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 95°C for 15 sec and 60°C for 1 min. To determine fold-

difference between samples additional reactions with 4 serial 5-fold dilutions of LNCaP cDNA were performed to generate a standard curve, which related the threshold cycle to the log input amount of template.

Chromosomal Mapping. Radiation hybrid mapping was done with the human GeneBridge 4 RH Panel (Research Genetics). The primers used were the same as the primers used for real time PCR. Results were submitted to WICGR Mapping Service by internet (www-genome.wi.mit.edu/cgi-bin/contig/rhmapper.pl) which gave us the computed results.

Transfections and Cell Proliferation Assays. cDNA coding for TMEFF2 was amplified from LNCaP cells with the following primes: 5'-CCGTTTGCTGCAGAG-TTGCACGAAC-3' and 5'-TCCAGCCACTGTGAAACATGCTCCC-3' and cloned into the pCDNA3 expression vector (Invitogen). DU 145 cells were transfected using the GenePORTER™ transfection Reagent (GTS Inc.) with 10 µg of either an empty pCDNA3.1 vector or the same vector expressing TMEFF2. Stable DU145 clones were obtained by selection in G418 (500 µg/ml). Expression of TMEFF2 was confirmed by Northern analysis. For MTT assays, DU145/neo (transfected with the empty vector) or DU145/TMEFF2 (transfected with the TMEFF2 expression vector) were seeded in quadruplicate in 96-well plates at 1000 cells per well. After 4 days, cell proliferation was assayed using the Cell Proliferation kit I (MTT, Boehringer Mannheim) according to the manufacture's instructions. For colony formation assays, PC3 cells were seeded in 6-well plates and transfected using the GenePORTER™ transfection Reagent (GTS Inc.) with 2.5 µg of either the empty pCDNA3.1 vector or the same vector expressing TMEFF2.

Transfected cells were grown in the presence of G418 (500 µg/ml) for 2 weeks and then stained with Giesma stain (Fisher Scientific).

Acknowledgements

This work was supported by National Institutes of Health Grant CA26038-20, Aron Eschman Fund, Parker Hughes Fund, C. & H. Koeffler Fund, Ko-So Foundation and Israel Humanitarian Fund. H. P. K. is the holder of the Mark Goodson Chair in Oncology and is a member of the Jonnson Cancer Center.

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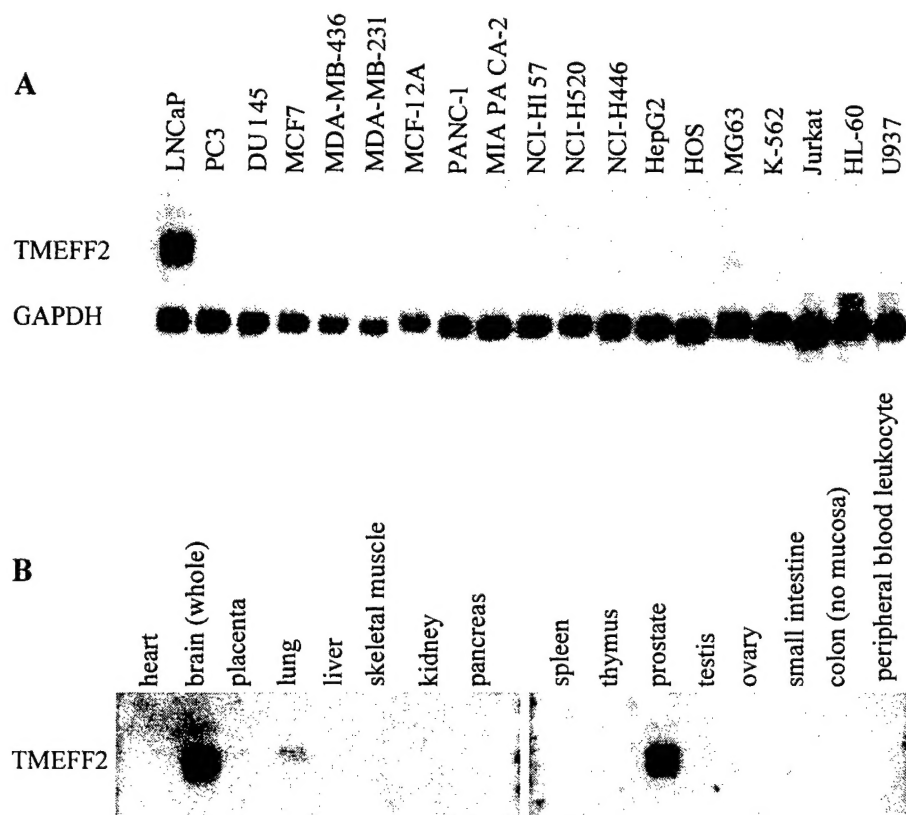


Figure 1

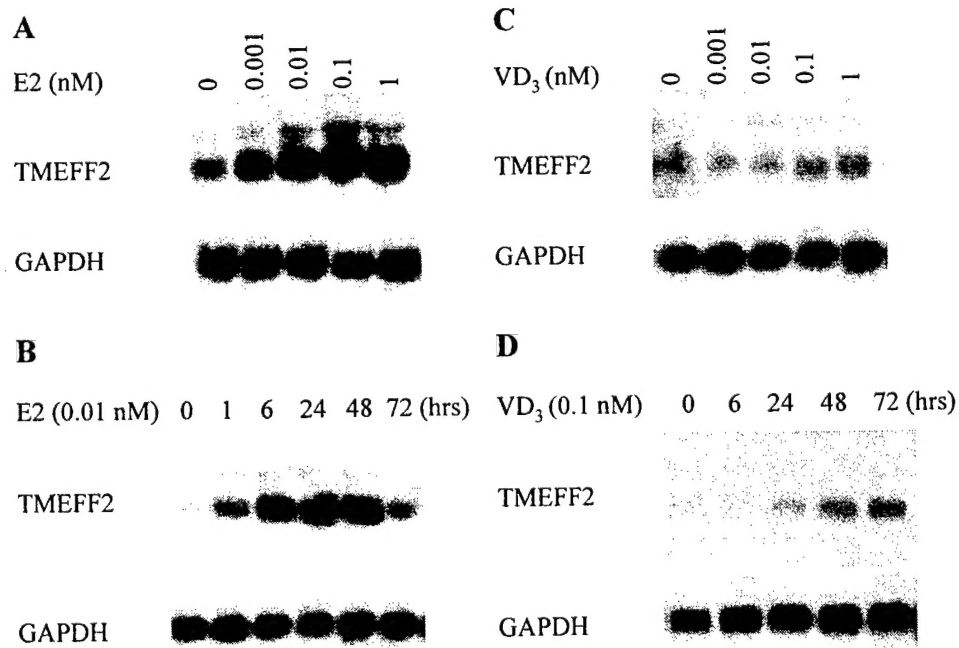


Figure 2

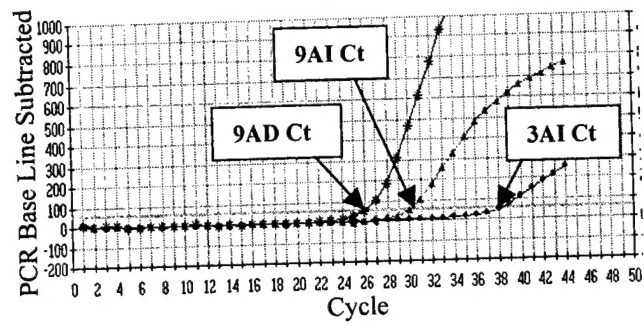


Figure 3

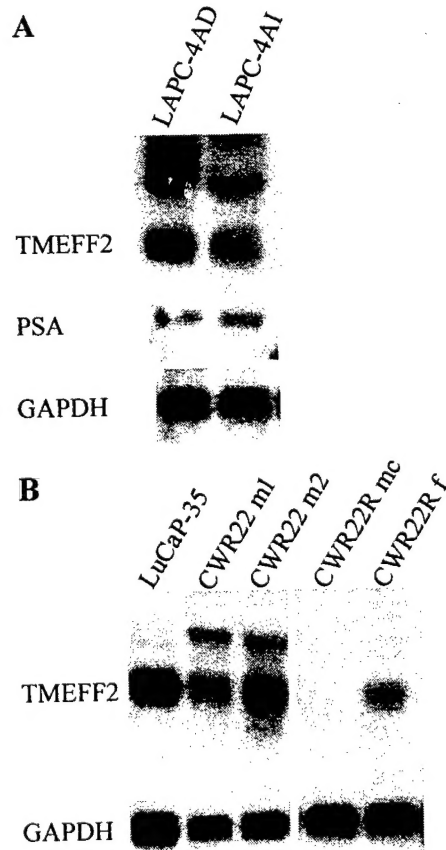


Figure 4

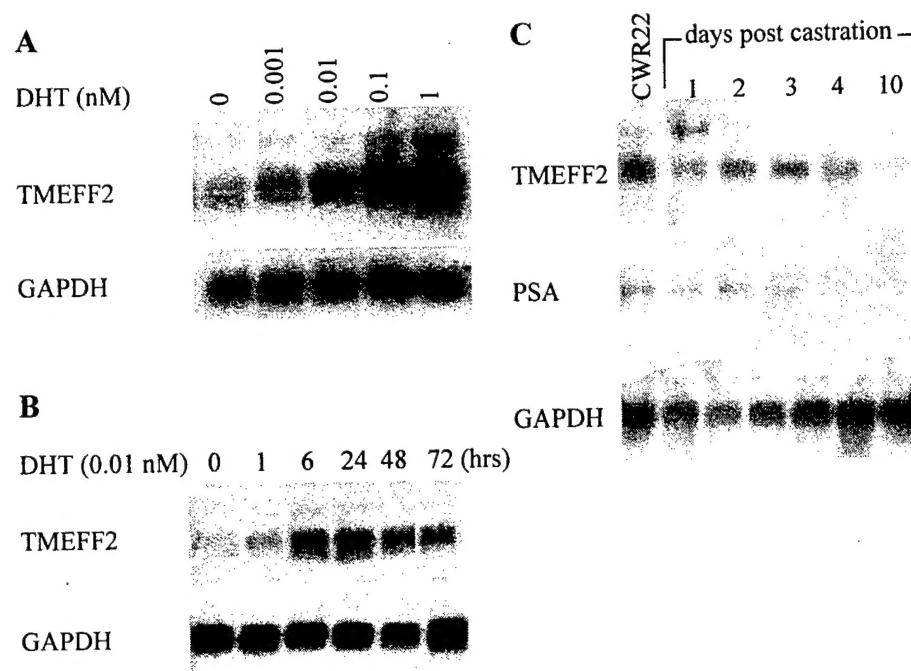


Figure 5

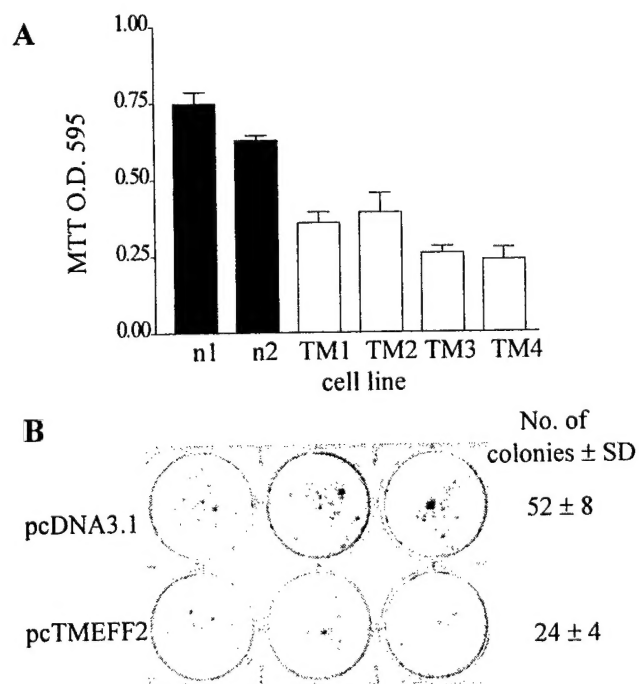


Figure 6

#3355 TMEFF2 is a New Member of the EGF-Like Family Regulated by Androgen and Having Antiproliferative Effects in Prostate Cancer Cells. Sigal Gery, Vijay Vegesna, Charles L. Sawyers, David B. Agus, and Phillip H. Koeffler. *Cedars-Sinai Medical Center/UCLA School of Medicine, Los Angeles, CA, and UCLA School of Medicine, Los Angeles, CA.*

Using subtractive hybridization, we identified a gene that was highly expressed in the androgen dependent (AD) prostate cancer cell line, LNCaP and undetectable in the androgen independent (AI) PC3 and DU145 cell lines. Sequence analysis revealed that it was identical to a newly cloned gene designated TMEFF2. TMEFF2 encodes a transmembrane protein containing an epidermal growth factor (EGF)-like motif and two follistatin domains. We mapped TMEFF2 to human chromosome 2q32.1. Extensive examination of normal and cancer human tissues for TMEFF2 mRNA expression by Northern blot demonstrated that it is expressed almost exclusively in prostate and brain. TMEFF2 mRNA was low in LNCaP cells grown in the absence of hormones, and markedly increased in a dose-dependent manner by dihydrotestosterone and 17β -estradiol. Furthermore, TMEFF2 expression was higher in the AD human prostate cancer xenografts, LAPC4-AD, LAPC9-AD and CWR22 than in their AI sublines (LAPC4-AI, LAPC9-AI and CWR22R). In addition, TMEFF2 levels decreased by day 10 post castration in the AD CWR22 xenografts. TMEFF2 expression vector was stably placed into DU145. These cells grew slowly in liquid culture and were much less tumorigenic as compared to wild type cells in nude mice. Our results demonstrate that TMEFF2 is an androgen responsive gene expressed in normal prostate cells as well as in AD prostate cancer cells and its forced expression inhibits tumor growth. Loss of expression of TMEFF2 occurs with transformation to AI suggesting it may act as a suppressor of prostate cancer progression to AI.